The 3Rs: reduction and refinement through a multivariate statistical analysis approach in a behavioural study to unveil anxiolytic effects of natural extracts of Tilia Tomentosa.

Guendalina Olivero[1], Federica Turrini[1], Raffaella Boggia[1], Paola Zunin[1], Dario Donno[2], Gabriele Loris Beccaro[2], Massimo Grilli[1,3], Anna Maria Pittaluga[1,3]

[1] Department of Pharmacy – DIFAR, University of Genoa, Viale Cembrano 4, 16148 Genoa, Italy

[2] Department of Agriculture, Forestry and Food Science, University of Torino, Largo Braccini, 2,10095 Grugliasco, Turin, Italy

[3] Inter-University Center for the Promotion of the 3Rs Principles in Teaching & Research

Bud-derivatives, obtained by macerating meristematic fresh tissues of trees and herbaceous plants, represent a relatively new class of botanicals. In the most countries of the EU, bud-derivatives, named also gemmoderivatives or embrioextracts, are classified as plant food supplements. In a recent paper Allio et al. (J Ethnopharmacol. 2015. 172:288-96) investigated the impact of *Tilia tomentosa* bud extracts (TTBEs) at Gamma-aminobutyric acid (GABA)ergic synapses by performing post-synaptic voltage-clamp recordings in hippocampal neurons. Direct application of TTBEs on post-synaptic terminals activated a chloride current in a way consistent with the activation of $GABA_A$ receptors. The involvement of these receptors was confirmed by the observations that either bicuculline and picrotoxin prevented the TTBEs-induced effects.

The aim of our work, supported by FINNOVER (n° 1198), the Interreg ALCOTRA Italy/France trans frontier project, was to investigate the effects of an "*in vivo"* oral administration of TTBEs on the behaviour of adult (3-6 months old) and aged (20-22 months old) male and female mice. In particular, we analysed the behavioural skills related to spontaneous motor activity, curiosity and anxiety in the hole-board maze and in the light-dark box. TTBEs (1÷2000 dilution) were dissolved in the drinking water and animals were monitored for the daily water intake and for the gain of weight. Behavioural tests were performed before and at the end of the TTBEs treatment.

We carefully planned this study according to the principles of reduction and refinement of the 3Rs.

In particular, any effort was made to minimize:

- the number of animals used in the experiments to obtain robust and reproducible results
- the number of behavioural tests to reduce stress in the animals.

The application of a strong multivariate analysis statistical approach allowed us to maximize the data obtained from each animal, refine the experimental plan and reduce the number of animals. The multivariate statistical analysis relied on chemometric steps such as unpattern recognition (i.e. PCA, Principal Component Analysis), variable selection (i.e. by genetic algorithms), modelling/classification techniques (i.e. SIMCA and UNEQ) and regression techniques.

Interestingly, our results show that the TTBE administration affects in a gender and in an age-dependent manner the curiosity, the anxiety and the spontaneous motor activity of the animals.

Our data suggest that TTBEs could be an interesting prototype of food supplements that can help to improve the well-being and to reduce the anxiety in humans and animals.